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Applicability of Monoclonal Antibody Fab Fragments as a Carrier of Neocarzinostatin in Targeting Chemotherapy

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Two types of fragments of MAb A7 were produced to improve the efficacy and safety in targeting chemotherapy with neocarzinostatin. In this study, ^{125}I-labeled F(ab')$_2$ and Fab fragments of MAb A7 and ^{125}I-labeled MAb A7 were injected intravenously into mice with pancreatic carcinoma xenografts, and the accumulation of each antibody in the tumors was compared. A greater amount of the ^{125}I-labeled Fab fragments of MAb A7 localized in the tumor 2 h following the injection than was observed with the other probes. Relatively less ^{125}I-labeled MAb A7 localized in the tumor 2 h following the injection than was observed with the other two probes. Moreover, reaction of rabbit antimouse IgG with the Fc portion, which is the most immunopotent region of the Fab and F(ab')$_2$ fragments of MAb A7 and MAb A7, was determined by ELISA; the weakest reaction was observed with the Fab fragments of MAb A7. These results suggest that the Fab fragments of MAb A7 may be more suitable carriers of an anticancer drug that is inactivated rapidly in the blood, such as NCS, in targeting chemotherapy than either intact MAb A7 or the F(ab')$_2$ fragments of MAb A7.

KEY WORDS: **pancreatic cancer, targeting chemotherapy, monoclonal antibody A7, Fab fragment, F(ab')$_2$ fragment, neocarzinostatin**

INTRODUCTION

Monoclonal antibodies (MAbs) against tumor-associated antigens have been shown to be potentially valuable in the diagnosis and treatment of human cancers [1–3], since hybridoma technology was developed by Köhler and Milstein [4]. The use of MAb–drug conjugates and MAb–toxin conjugates for solid tumors have been reported [5–7]. This has been made possible by the availability of MAbs that recognize the cell surface antigens of various carcinomas. The objective of targeting chemotherapy is to increase the therapeutic effect of the cytotoxic agent by enhancing its localization in the target tumor.

In our laboratory, we developed MAb A7 [8] and covalently cross-linked it to an anticancer antibiotic, neocarzinostatin (NCS), to form a MAb–drug conjugate (A7-NCS) [9]. A7-NCS has been used for treating patients with colorectal carcinoma and has yielded promising results [10]. In addition, we have reported that A7 antigens are expressed in a high percentage of human pancreatic carcinomas, and the A7 antigens are shed rarely into the blood from the cancer cells [11]. However, when A7-NCS was administered to mice with human pancreatic cancer xenografts, the tumors were able to grow in the presence of A7-NCS, even though A7-NCS showed greater antitumor activity than did free NCS [12].

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Recently, fragments of various MAbs have been investigated for tumor imaging. Several types of Mab fragments, particularly the $F(ab')_2$ and Fab fragments, are regarded generally as yielding improved tumor imaging [13,14]. This is due possibly to their faster rate of extravasation and penetration of target tumor tissues. Thus, MAb fragments may be suitable as the carriers of anticancer agents that are inactivated rapidly in the blood.

In this study, we demonstrated improved rapid tumor localization of the Fab fragments of MAb A7 and investigated the possibility that these fragments can be used for targeting chemotherapy against human pancreatic cancer.

MATERIALS AND METHODS

Cell Line

The human pancreatic carcinoma cell line HPC-YS [11] was used in this study. HPC-YS cells were established from a ductal cell adenocarcinoma of the human pancreas and obtained from Dr. N. Yamaguchi (Research Institute of Neurology and Geriatrics, Kyoto Prefectural University of Medicine). These cells can be maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories, Rockville, MD).

Tumor Xenografts

Approximately 5×10^6 viable HPC-YS cells were injected subcutaneously (s.c.) into the left flank of athymic 8-week-old male nude mice (BALB/C, nu-nu) (SLC Co., Shizuoka, Japan). A tumor mass was detected in all mice injected with the HPC-YS cells.

MAbs

MAb A7 [8] is an IgG_1, and has been reported to react with 77% of human pancreatic carcinomas tested, as well as with 70% of human colonic carcinomas [12]. The MAb A7 does not react immunohistochemically with normal pancreatic tissues [15]. Normal mouse IgG_1 was purchased from Boehringer Mannheim Biochemical (Mannheim, Germany).

Fragments of MAbs

Pepsin was added to the MAb A7 in a ratio of 1:47 (weight/weight) in 0.1 M sodium acetate buffer, pH 3.7, and incubated at 37°C for 5 h. After the reaction had been stopped by the addition of 5 N NaOH, the $F(ab')_2$ fragments were separated by high-performance liquid chromatography (HPLC). To produce the Fab fragments of MAb A7, papain (16–40 U/mg, Sigma) was added to MAb A7 in a ratio of 1:100 (weight/weight) in 0.1 M phosphate buffer containing 0.01 M 2-mercaptoethanol, pH 7.2, and incubated at 37°C for 7 h. After the reaction had been stopped by the addition of 0.014 M iodoacetamide, the preparations were dialyzed in 5 mM disodium hydrogenphosphate buffer. The Fab fragments were separated immediately by ion-exchange chromatography and gel filtration. The $F(ab')_2$ and Fab fragments of MAb A7 retained a binding activity nearly identical to that of intact MAb A7 [16,17]. The $F(ab')_2$ and Fab fragments of normal mouse IgG were purchased from Cappel (Rockland, PA).

Radiolabeling of MAbs and $F(ab')_2$ and Fab Fragments

The MAb A7, the $F(ab')_2$, and Fab fragments of MAb A7, normal mouse IgG, and the $F(ab')_2$ and Fab fragments of normal mouse IgG were radiolabeled with ^{125}I (Amersham Japan, IMS 30, Japan) using the chloramine-T method [18]. The iodinated MAb A7 was separated from excess reactants by gel filtration on a Sephadex G-25 column. The MAb A7, the $F(ab')_2$, and Fab fragments of MAb A7, normal mouse IgG, and the $F(ab')_2$ and Fab fragments of normal mouse IgG were labeled with ^{125}I to specific activities of 7.2, 7.8, 6.0, 8.0, 7.5, and 8.0 μCi/μg, respectively.

Distribution of ^{125}I-Labeled MAb A7, $F(ab')_2$, and Fab Fragments of MAb A7

The tumor localization of the MAb A7 and the $F(ab')_2$ and Fab fragments of MAb A7 was investigated in athymic nude mice bearing HPC-YS tumors and compared to that of normal mouse IgG, and the $F(ab')_2$ and Fab fragments of normal mouse IgG. Three weeks following inoculation, the tumor-grafted mice were divided into six groups; 16 mice were injected intravenously with 0.7 μCi of either ^{125}I-labeled MAb A7, ^{125}I-labeled normal mouse IgG, ^{125}I-labeled $F(ab')_2$ fragments of MAb A7, ^{125}I-labeled $F(ab')_2$ fragments of normal mouse IgG, ^{125}I-labeled Fab fragments of MAb A7, or ^{125}I-labeled Fab fragments of normal mouse IgG. Four mice from each group were killed at 2, 6, 12, and 24 h following injection, and the tumors and blood from each mouse were collected and weighed. The mean weight of the tumors was 180 mg. The amount of radioactivity in each tissue specimen then was measured using a γ-scintillation counter. The result from each tissue was expressed as cpm/g and compared with the other groups. To compare the kinetics of the localization of the three probes in the tumor and blood, the percentage injected dose/g (%ID/g) was calculated. To compare the accumulation of ^{125}I-labeled MAb A7, ^{125}I-labeled $F(ab')_2$ fragments of MAb A7, and ^{125}I-labeled Fab fragments of MAb A7 in the normal tissues, the normal tissues were collected at 2 h following the injection and %ID/g was calculated. Furthermore, to compare the specific localization of the six probes in the tumors to that in the blood, the ratio of the radioactivity in the tumor to that in the blood was calculated. These ratios were derived by dividing the radioactivity per unit weight of the tumors by that in the blood per unit weight. Student's *t*-test was used to check for statistically significant differences.

Antigenicity of MAb A7 and Its Fragments

An enzyme-linked immunosorbent assay (ELISA) was performed against MAb A7, and the $F(ab')_2$ and Fab fragments of MAb A7. Various concentrations of MAb A7, and the $F(ab')_2$ and Fab fragments of MAb A7 in carbonate–bicarbonate buffer, pH 8.4 were seeded in 96-well microtiter plates (Flow Laboratories, MD) and incubated at 37°C for 2 h. Following five washes with 0.05% Tween 20 in phosphate-buffered saline (PBS), the wells were treated with 25% Block Ace (Dainippon Kagaku, Japan) in PBS. Following removal of the Block Ace, the wells were incubated with 100 μl of peroxidase-labeled rabbit antimouse IgG antibody (Zymed Laboratories, CA), which reacts with the Fc portion of mouse IgG, for 30 min. Following five rinses with 0.05% Tween 20 in PBS, 100 μl of the substrate solution consisting of 0.1 M citrate buffer, pH 4.0 containing azinodiethylbenzolin (ABTS) (0.5 mg/ml), and 0.01% H_2O_2 was added to each well. The absorbance was read at 414 nm by an automatic immunoreader (BioRad, CA).

RESULTS

Distribution of ^{125}I-Labeled MAb A7, $F(ab')_2$ and Fab Fragments of MAb A7

We found that 3.50 ± 0.77 (mean ± SD) %ID/g of ^{125}I-labeled MAb A7 localized in the tumor at 2 h following the injection and increased with time. The radioactivity reached 6.12 ± 0.79 %ID/g in the tumor at 24 h following the injection. By contrast, the tumor accumulation level of ^{125}I-labeled normal mouse IgG was 3.05 ± 0.83 at 2 h following the injection and decreased monotomically with time, while 3.85 ± 0.85 %ID/g of the ^{125}I-labeled $F(ab')_2$ fragments of MAb A7 localized in the tumor at 2 h following the injection and increased with time until 12 h following the injection. As for ^{125}I-labeled $F(ab')_2$ fragments of normal mouse IgG, the tumor accumulation level was 2.55 ± 0.40 %ID/g at 2 h following the injection and decreased with time. The %ID/g of ^{125}I-labeled Fab fragments of MAb A7 that accumulated in the tumor 2 h following the injection was 4.34 ± 0.93 and then decreased with time to 2.10 ± 0.53 at 24 h following the injection. The tumor accumulation values of the ^{125}I-labeled Fab fragments of normal mouse IgG were lower than any other probe antibody fragments at any time points (Fig. 1).

The ^{125}I-labeled MAb A7 and normal mouse IgG persisted longer in the blood than the other antibody fragments. By contrast, the ^{125}I-labeled Fab fragments of MAb A7 and normal mouse IgG were cleared more rapidly than any the other probe antibody fragments (Fig. 2).

The tumor tissue/blood ratio of the radioactivity of the Fab fragments of MAb A7 was 1.00 ± 0.12 (mean ± SE) at 2 h following the injection and increased rapidly with time. The tumor tissue/blood ratio of the $F(ab')_2$ fragments of MAb A7 and whole MAb A7 was 0.25 ± 0.03 and 0.12 ± 0.012 2 h following the injection, respectively, and increased with time. The tumor tissue/blood ratio of the radioactivity of the Fab fragments of MAb A7 was significantly higher than that of the $F(ab')_2$ fragments of MAb A7 or the MAb A7 (Fig. 3).

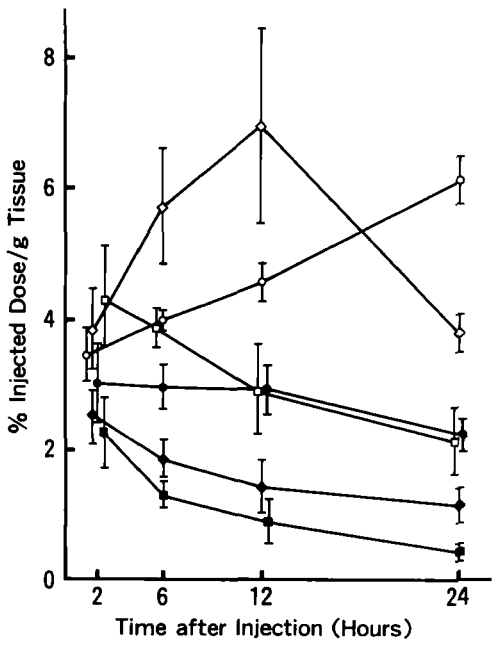

Fig. 1. Concentration of ^{125}I-labeled MAb A7 and ^{125}I-labeled $F(ab')_2$ and Fab fragments of MAb A7 in HPC-YS tumors in mice following an intravenous injection. More of the ^{125}I-labeled Fab fragments of MAb A7 localized in the tumor than did the other probes 2 h following the injection. The least amount of ^{125}I-labeled MAb A7 localized in the tumor 2 h following the injection. ○, ^{125}I-labeled MAb A7; ●, ^{125}I-labeled normal mouse IgG; ◇, ^{125}I-labeled $F(ab')_2$ fragments of MAb A7; ◆, $F(ab')_2$ fragments of normal mouse IgG; □, ^{125}I-labeled Fab fragments of MAb A7; ■, ^{125}I-labeled Fab fragments of normal mouse IgG; points, means; bars, SD.

Figure 4 shows the accumulation of ^{125}I-labeled MAb A7, ^{125}I-labeled $F(ab')_2$ fragments of MAb A7, and ^{125}I-labeled Fab fragments of MAb A7 in the normal tissues at 2 h following injection. More ^{125}I-labeled ^{125}I-labeled Fab fragments of MAb A7 accumulated in the kidney than MAb A7 and ^{125}I-labeled $F(ab')_2$ fragments of MAb A7 at 2 h following the injection.

Antigenicity of MAb A7 and Its Fragments

Of the three MAb A7 probes, MAb A7 reacted most strongly with rabbit antimouse IgG antibody. In contrast, the Fab fragments of MAb A7 reacted the least with rabbit antimouse IgG antibody (Fig. 5).

DISCUSSION

We have reported that MAb A7 can be linked covalently to NCS and that this conjugate can be used to treat patients with colorectal cancer. Some patients who have

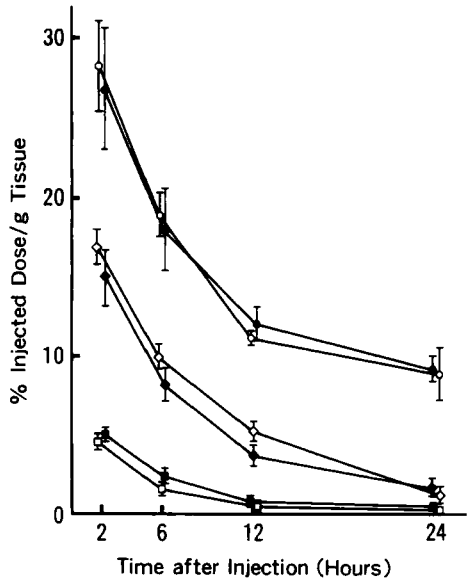

Fig. 2. Concentration of ^{125}I-labeled MAb A7 and ^{125}I-labeled F(ab')$_2$ and Fab fragments of MAb A7 in the blood of mice following an intravenous injection. The serum concentration of the ^{125}I-labeled Fab fragments of MAb A7 decreased more rapidly with time than that of either the ^{125}I-labeled F(ab')$_2$ of MAb A7 or the ^{125}I-labeled MAb A7. The serum concentration of the ^{125}I-labeled MAb A7 was much higher than that of either the ^{125}I-labeled F(ab')$_2$ or the Fab fragments of MAb A7. ○, ^{125}I-labeled MAb A7; ●, ^{125}I-labeled normal mouse IgG; ◇, ^{125}I-labeled F(ab')$_2$ fragments of MAb A7; ◆, F(ab')$_2$ fragments of normal mouse IgG; □, ^{125}I-labeled Fab fragments of MAb A7; ■, ^{125}I-labeled Fab fragments of normal mouse IgG; points, means; bars, SD.

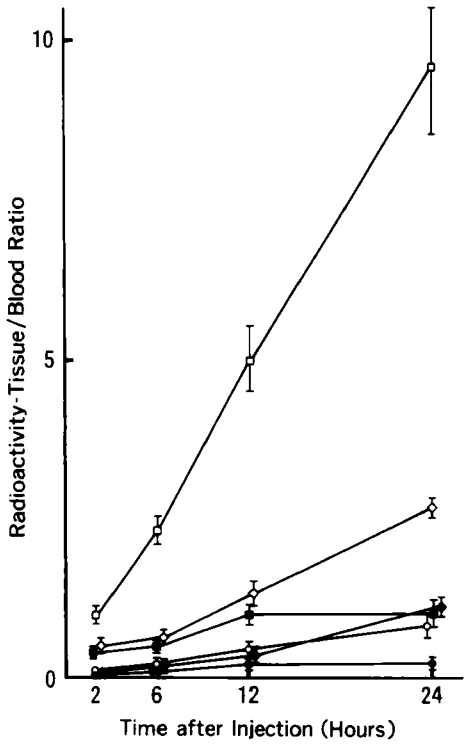

Fig. 3. Tumor tissue/blood ratio of the MAb A7, F(ab')$_2$ and Fab fragments of MAb A7. The tumor tissue/blood ratio of the radioactivity of the Fab fragments of MAb A7 increased more rapidly with time than that of either the F(ab')$_2$ fragments of MAb A7 or whole MAb A7. ○, ^{125}I-labeled MAb A7; ●, ^{125}I-labeled normal mouse IgG; ◇, ^{125}I-labeled F(ab')$_2$ fragments of MAb A7; ◆, F(ab')$_2$ fragments of normal mouse IgG; □, ^{125}I-labeled Fab fragments of MAb A7; ■, ^{125}I-labeled Fab fragments of normal mouse IgG; points, means; bars, SE.

been treated with this conjugate have had a partial regression of their tumors [10]. We have also reported that the A7-NCS conjugate showed greater antitumor activity against human pancreatic cancer than did an NCS solution. The antitumor effect of NCS was thought to be enhanced due to the antigen–antibody bindings of MAb A7, because a greater quantity of ^{125}I-labeled MAb A7 than ^{125}I-labeled normal mouse IgG localized in the tumor. However, the tumor growth was not suppressed completely with the A7-NCS treatment. The main reason for the inadequate antitumor effect of A7-NCS on human pancreatic tumors may be the low level of active NCS within the tumors. Fujita et al. [19] have reported that more than 70% of the administered NCS is inactivated by serum within 120 min. As shown in Figure 1, less ^{125}I-labeled MAb A7 localized in the tumor than any of the fragments of MAb A7 at 2 h following the injection. The Fab and F(ab')$_2$ fragments of MAb A7 showed specific localization in the tumors, since more of these fragments than normal mouse IgG accumulated in the tumors. In general, the small variable fragment of the MAb molecule, when labeled with a radionuclide, has the ability to leave the vascular space rapidly and to penetrate target tumor tissue easily. Thus, the Fab fragments of MAb A7 may be able to carry a large amount of a short-acting anticancer drug like NCS because the molecular weight of the Fab fragments is smaller than that of intact immunoglobulin or the F(ab')$_2$ fragments.

Recent research has been directed toward the use of the Fab or F(ab')$_2$ fragments of immunoglobulins for the immunodetection of solid tumors [13,14]. These studies have demonstrated that the fragments of MAbs against tumor cells exhibit a higher tumor tissue/blood ratio of radioactivity than the intact MAbs because of their faster tumor localization and blood clearance. We also have reported that the Fab and F(ab')$_2$ fragments of MAb A7 are useful in the tumor imaging of pancreatic cancer [16,17]. In the present study, the ^{125}I-labeled Fab fragments disappeared more rapidly than the other antibody fragments. Because of their rapid clearance, the use of the Fab fragments of MAbs as carriers of anticancer drugs minimizes their toxic effects, which represent a major limitation in conventional chemotherapy. Hansson et al. [20] have demonstrated, however, that non-Fc-bearing antibody fragments are cleared rapidly from the blood and that this rapid clearance is thought to occur mainly via the kidney, while the removal of intact Ig occurs mainly through interactions with Fc-receptor bearing cells, followed by their slow clearance via the reticuloen-

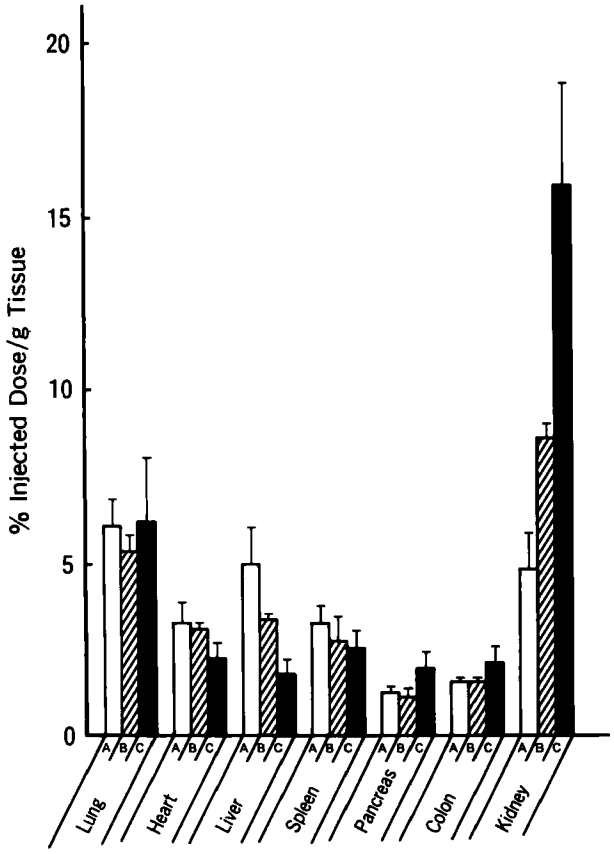

Fig. 4. Concentration of ^{125}I-labeled MAb A7 and ^{125}I-labeled F(ab')$_2$ and Fab fragments of MAb A7 in the normal tissues in mice at 2 h following the injection. More of the ^{125}I-labeled Fab fragments of MAb A7 localized in the tumor than did the other probes 2 h following the injection. More ^{125}I-labeled Fab fragments of MAb A7 accumulated in the kidney than ^{125}I-labeled MAb A7 and ^{125}I-labeled F(ab')$_2$ fragments of MAb A7. A, ^{125}I-labeled MAb A7; B, ^{125}I-labeled F(ab')$_2$ fragments of MAb A7; C, ^{125}I-labeled ^{125}I-labeled Fab fragments of MAb A7; bars, SD.

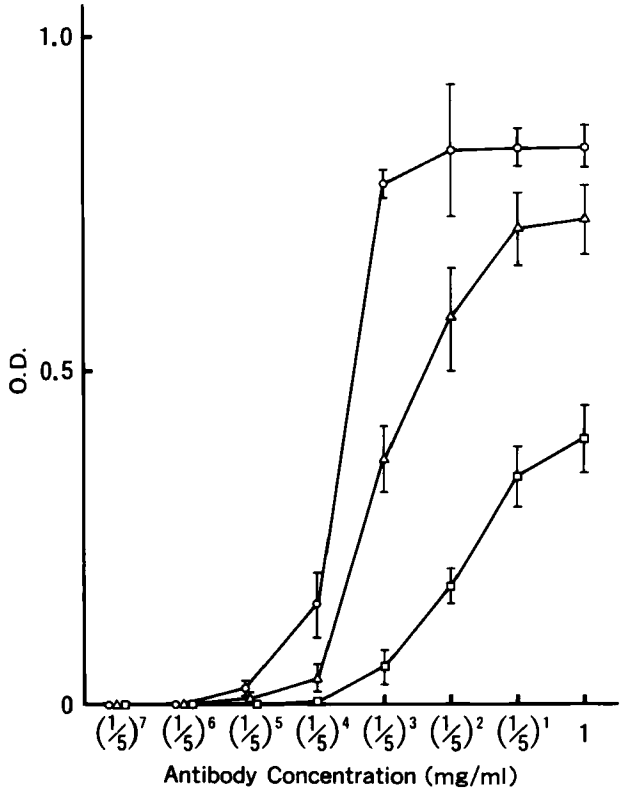

Fig. 5. ELISA for the antigenicity of the MAb A7 and the F(ab')$_2$ and Fab fragments of MAb A7 using rabbit antimouse immunoglobulin. The Fab fragments of MAb A7 reacted the least well with the rabbit antimouse IgG antibody. ○, MAb A7; △, F(ab')$_2$ fragments of MAb A7; □, Fab fragments of MAb A7; points, means; bars, SD.

dothelial system. In this study, the localization of the Fab fragments of MAb A7 in the kidney was relatively high at 2 h following the injection. However, Fujita et al. [19] have reported that NCS is inactivated rapidly in the kidney. Therefore, the toxic effects of NCS on the kidney may be minimized if NCS conjugated to Fab fragments are administered.

Murine MAbs administered to humans induce a human antimouse antibody (HAMA) response [21–23] that may reduce the tumor localization of the MAb and lead to anaphylactic reactions. Takahashi et al. [10] reported previously that HAMA was produced in all patients who received A7-NCS. Because the Fc portion of an immunoglobulin is the most immunopotent region of intact MAbs [24], the immunologic advantage of Fab fragments over intact MAbs is that the Fab fragments lack the Fc portion of the molecule. We could not evaluate for any differences in the antigenicity of intact MAb A7, and the F(ab')$_2$ and Fab fragments of MAb A7 to humans in these experiments because we were unable to prepare a human antimouse antibody. However, in the immunologic study using rabbit antimouse IgG, the reaction of rabbit antimouse IgG with the Fc portion which is the most immunopotent region of MAbs was the lowest in the Fab fragments of MAb A7, while that of intact MAb A7 was the highest.

This study suggested that the Fab fragments of MAb A7, which are useful for immunoimaging, may be a more suitable carrier of NCS against human pancreatic cancer in targeting chemotherapy than either intact MAb A7 or the F(ab')$_2$ fragments of MAb A7.

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